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Physiological and molecular response of *Lactuca sativa* to colonization by *Salmonella enterica* serovar Dublin

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Summary

This paper describes the physiological and molecular interactions between the human pathogenic *Salmonella enterica* serovar Dublin and the commercially available mini Roman lettuce cv Tamburo. The association of *S.* Dublin with lettuce plants was first determined, which indicated the presence of significant populations outside and inside the plants. The latter was evidenced from significant residual concentrations after highly efficient surface disinfection (99.81%) and fluorescence microscopy of *S.* Dublin in cross-sections of lettuce at the root-shoot transition region. The plant biomass reduced significantly compared to that of non-colonized plants upon colonization with *S.* Dublin. Next to the physiological response, also transcriptome analysis by cDNA-AFLP provided clear differential gene expression profiles between non-colonized and colonized lettuce plants. From this generally and differentially expressed genes were selected and identified by sequence analysis, followed by RT-PCR displaying the specific gene expression profiles in time. Functional grouping of the expressed genes indicated a correlation between colonization of the plants and an increase in expressed pathogenicity-related genes.

This study indicates that lettuce plants respond to the presence of *S.* Dublin at a physiological and molecular level as shown by the reduction in growth and the concurrent expression of pathogenicity-related genes. In addition, it was confirmed that *Salmonella* spp. can colonize the interior of lettuce plants, thus potentially impose a human health risk when processed and consumed.

Introduction

In recent years an increase in bacterial foodborne disease outbreaks has been associated with the consumption of uncooked vegetables (i.e. 1, 14, 21, 30, 32). The economical impact of these outbreaks is large, for example each year *Salmonellosis* is responsible for 3.5 million cases in the US and Canada, leading to economical losses up to 3.4 billion $ a year (41). Especially bacterial pathogens like *S. enterica* (17), *Escherichia coli* O157:H7 (39), *Bacillus cereus* (7), *Listeria monocytogenes* (37), *Campylobacter jejuni* (7), *Pseudomonas* spp.(19, 44) are of major concern due to the environmental occurrence of these bacteria. The presence of human pathogenic bacteria has been described on a wide range of plant hosts (7, 17, 18, 19, 21, 34, 37, 39, 44, 47). For greenhouse grown vegetables, these pathogens are suggested to be introduced as a result of bad hygiene during the production or post-harvest processing of the crops (1, 7). However, contamination of vegetables may already occur in the field when manure is used for soil fertilization before planting the seedlings (34, 44). Manure is known to harbor high numbers of human pathogenic bacteria like *Salmonella* spp. and *E.coli* O157, which can remain viable for extensive periods of time, even up to one year (5, 27, 45). Even when applying artificially contaminated manure to soil, the number of enteric bacteria was reduced only 1 order of magnitude after a period of three months (15). Thus, contamination of plants with human pathogenic bacteria from manure may occur, for example during rainfall or irrigation due to splashing of soil and bacteria onto the plants (34). Alternatively, plants could be colonized via the roots in manure-amended soil (39, 47). The colonization of plants via the roots by human pathogenic *E. coli* was observed using a gfp-tagged strain of *E.coli* O157:H7 that colonized the interior of lettuce from soil via the roots up to the leaves (16, 39). In contrast to this, two other studies found *E.coli* O157:H7 not able to colonize the edible parts of spinach (22) or crisphead lettuce (25), although the bacteria were detected in the rizosphere and on the root surface. With respect to *Salmonella* spp., gfp-tagged strains colonized the interior of tomato plants when grown hydroponically (17, 18). Also, an avirulent strain of *S.* Typhimurium colonized carrots and radishes which were grown on a field treated with contaminated manure composts or irrigation water (24). Just recently, *S.* Typhimurium LT2 and DT104h were

found to endophytically colonize barley sprouts during growth in an axenic system (28). FISH analysis of radial slices indicated the presence of *S.* Typhimurium inside the plant tissue. However, only very few studies have investigated the physiological effect or molecular interaction between human bacterial pathogens and a plant host, i.e. the model plants *Medicago* and *Arabidopsis*. On *A. thaliana* it was shown that the opportunistic human pathogen *Pseudomonas aeruginosa* PA14 attached to the leaf surface, congregated at the stomata or wounds, and then invaded the leaves and colonized the intercellular spaces (35). The bacterium was also able to make circular perforations in mesophyllic cell walls to allow penetration. From this study it was concluded that *Pseudomonas aeruginosa* PA14 is a facultative pathogen of *A. thaliana* that can cause local and systemic infection, eventually leading to plant death. Also mutants of the human pathogenic *Staphylococcus aureus* (36) that contained disrupted genes involved in animal pathogenesis, were attenuated in their ability to infect *A. thaliana*. This suggested that the same regulators that mediate synthesis of virulence factors essential for animal pathogenesis are also required for plant pathogenesis (36). Resistance of *A. thaliana* to *S. aureus* was mediated by a direct effect of salicylic acid on the pathogen affecting attachment on the root surface and reducing pathogen virulence.

Different *Salmonella* spp. were able to endophytically and epiphytically colonize *M. sativa* (11). A recent study revealed that colonization of *M. truncatula* by *S.* Typhimurium resulted in the induction of salicylic acid –dependent and –independent plant defenses (23). From this the induction of both plant defense pathways was correlated to the bacterial gene expression of TTSS-SPI effector proteins, whereas the presence of flagella only induced the SA-dependent plant defense induced by expression of the PR1-gene.

Although these studies give direction to a specific host-pathogen interaction, until now no research has been described studying the gene-expression of plants during colonization by human pathogenic bacteria, such as *Salmonella* spp.

The objectives of this study were to investigate the physiological and molecular response of *L. sativa* by *S.* Dublin during plant colonization. Colonization of lettuce plants by *S.* Dublin was studied

by comparing the prevalence and the degree of colonization on surface-disinfected and untreated plants grown in nutrient water-agar and in manure-amended soil. Epiphytic and endophytic presence of *S.* Dublin was investigated to provide insight in the capability of *S.* Dublin to invade plant tissue and to proliferate in or on the plant. To reveal generally and differentially expressed genes upon colonization of lettuce with *S.* Dublin in time, cDNA-AFLP gene-expression profiling was studied. Transcript derived fragments were subjected to sequence analysis and grouped by gene function. Subsequent gene-expression profiling of selected genes was performed using RT-PCR.

Materials and Methods

Plant material and bacterial strains

Seeds of *Lactuca sativa* cultivar *Tamburo* (mini-Roman lettuce) were kindly provided by Mr. Raats (Nickerson-Zwaan BV, The Netherlands). The seeds were surface-sanitized by washing with 1% sodium hypochloride / 0.01% Tween 20, and water (twice), for 1 min each. Subsequently the seeds were air-dried for 1 hour.

A liquid culture of *Salmonella enterica* serovar Dublin grown overnight at 30°C in tryptic soy broth, was kindly provided by Dr. H. Aarts (RIKILT, The Netherlands). The culture was maintained by both plating on selective Hektoen enteric agar (Biotec Laboratories Ltd., UK) and overnight incubation in buffered peptone water (BPW) at 37°C. An *Escherichia coli* JM109 culture (obtained from the collection of Plant Research International BV) was maintained on Luria Broth (LB) plates and in liquid LB medium by overnight incubation at 37°C.

Surface disinfection of lettuce plants colonised with Salmonella Dublin

To determine the efficiency of surface disinfection, 35 six weeks-old lettuce plants (grown on soil) were inoculated with 20µl of $2x10^8$ CFU / ml of *S.* Dublin. In total 10 µl of the inoculum was spread across the surface of one leaf and 10 µl was spread across the bottom of another leaf. After 5 min incubation at room temperature the plants were cut at the transition point and from 25 plants the leafy parts were disinfected by rinsing for 10 sec in 70% ethanol and twice in water. Subsequently, each plant (leafy part) was ground in 1 ml of BPW and a dilution series (100x and 1000x diluted) was prepared from the suspension. Each dilution was plated (40µl) on Hektoen enteric agar, in duplicate, and incubated overnight at 37°C prior to colony counting. The means and standard errors of the number of *Salmonella* CFUs recovered were calculated, and the surface disinfection efficiency was determined by the ratio between the mean [number of CFUs recovered from surface-disinfected plants] and the mean [number of CFUs recovered from non-disinfected plants].

Association of Salmonella Dublin with lettuce grown in manure-amended soil

Fresh manure was collected from a Dutch organic dairy farm. Soil was collected from a field (60 kg of top layer of 20 cm) from the organic experimental farm the Droevendaal (Wageningen, the Netherlands). The soil consisted of 89% sand, 8% silt, 3% clay, a total nitrate (N) and carbon (C) of 2135 mg / kg and 22400 mg / kg, 11% moisture and had a pH of 7.14. The manure contained 28.7% acid detergent fibre, 40.3% neutral detergent fibre, a total dissolved organic N and C of 740 mg / kg and 8167 mg / kg, 220 mg / kg ammonium, 8.14 mg / kg nitrate and had a pH of 6.8. Both substrates tested negative for presence of *Salmonella* spp., which was determined by plating directly on selective Hektoen enteric agar and by testing the total DNA extracts from 10 ml BPW enrichments of three random samples of 1g of each substrate using real-time PCR analysis (26). Manure was inoculated with 10^8 CFU of *S.* Dublin / g wet weight and mixed thoroughly before addition to soil at a weight ratio of 1:10. The final number of *S.* Dublin CFU was10^7 / g fresh mixture. In total 74 pots of 50 ml with 50 g of *S.* Dublin contaminated soil / manure mixture were prepared. The negative control pots (74 in total) contained non-*S.* Dublin-inoculated manure / soil mixture. One lettuce seed was added to each pot (148 in total) and allowed to germinate in a greenhouse at 18℃ and 80% humidity. After 6 weeks, each plant was harvested by cutting the plant at the stem just above the soil. The plants were each weighed and thoroughly washed in 30 ml of sterile water prior to analysis. Next, for both treatments all plants were randomly divided in two sets of 38 plants. Each plant of the first set of plants was ground in 1 ml of BPW. From the second set of plants each plant was surface disinfected as previously described, followed by grinding in 1 ml of BPW. Each suspension of ground plant material was plated (40µl) on Hektoen enteric agar, in duplicate. In addition, the wash fraction was centrifuged and the pellet resuspended in 100µl of BPW prior to plating on Hektoen enteric agar, in duplicate (40 µl / plate). After overnight incubation at 37℃ the total number of Salmonella CFUs was counted for each plate. To determine a significant difference in plant weight between both treatments, a paired t-test was performed for all tested plants per treatment.

Association of S. Dublin with lettuce grown on Hoagland's agar

Sterilized lettuce seeds (120) were allowed to germinate for 3 weeks on 0.5% Hoaglands agar (pH 6.8) in closable growing units (10 x 15 x 8 cm) placed in a growth chamber at 20°C with 12 hrs light / dark intervals. To assess the colonization of lettuce by *S.* Dublin over time, the 120 lettuce 3 weeks-old plants were inoculated at the root site with 10µl of 10^7 CFU / ml of *S.* Dublin, without wounding the roots. Every two days for a period of twenty days, the shoots of 12 lettuce plants were cut off just above the agar and weighed. To determine the prevalence, degree of colonization, and localization of *S.* Dublin (endophytically or epiphytically) associated with lettuce plants, the harvested leafy parts of six plants were not surface disinfected whereas leafy parts of the other six plants were surface disinfected as described earlier. The leafy parts were ground in 0.5 ml of BPW. A dilution series (non-diluted, 10x or 100x diluted) was prepared from each leaf suspension and 40µl of each dilution was plated onto Hektoen enteric agar, in duplicate. With both treatments (with and without surface disinfection) the prevalence and the degree of colonization was determined by calculating the mean *S.* Dublin CFU subtracted by the error of surface disinfection efficiency.

Lettuce response to bacterial colonization

The response of lettuce plants to colonization by *S.* Dublin was compared to that by *E. coli* JM109. Water-inoculated plants were used as controls. Thirty seeds were sprouted on sterile Hoagland agar in separate tubes in a closable growing unit (50 x 30 x 25 cm) for 3 weeks in a growth chamber at 20°C and 80% humidity. Next, each 10 sprouts were carefully inoculated at the roots with 10µl of 10^7 CFU / ml of *S.* Dublin, 10µl of 10^7 CFU / ml of *E. coli* JM109, or inoculated with water. After 5 weeks, each surviving plant per treatment was cut at the transition point between stem and roots and weighed. To determine significant differences between the treatments with respect to plant death, non-parametric analysis (Kruskal-Wallis test with asymptotic significance) were performed based on the number of surviving plants and the weight of these plants.

Preparation of plant tissue cross-sections

From a different set of lettuce plants that were inoculated in a similar manner as described above, 3 weeks after inoculation with *S.* Dublin the transition region was cut for microscopic analysis before

cross-sectioning (in total 1 cm of transition region was obtained for each plant). First, the cut transition regions were incubated overnight in fixative (Ethanol 96% : Acetic acid (3:1 v/v)). After fixation the tissues were transferred to a graded series of sucrose solutions with increasing concentrations of 5, 10, 20, 30, 40 and 50% (w/v) in PBS (phosphate buffered saline, pH 7.4). Samples were kept in each concentration for 30 min. Next, the samples were embedded in Tissue-Tek O.C.T compound (Miles, Elkhart, In) while ensuring a vertical position of the samples. Cross sections of 20 µm and 200µm thickness were cut from each sample using a cryostat (Microm, HM 500 O, Microm Laborgeräte GmbH, Walldorf, Germany) at -30°C. The tissue sections were transferred to poly-l-lysine (Sigma Chemicla Co, St. Louis, MO, p-1524; 0.1% in milliQ (w/v)) coated slides and dried at 60°C for 15 min and stored at -20°C upon further use. To label the potentially present *S.* Dublin in each cross section, the slides were first incubated at 70°C for 15 min, washed once with milliQ (without shaking) at RT, dried at 70°C, washed twice with milliQ at RT, dried at 70°C, washed with PBST (PBS including 0.1% Tween) for 2 min at RT, washed with PBS with 2% bovine serum albumin (BSA) for 10 min and washed twice with PBST at RT. Next, the cross sections were incubated in the dark for 60 min at RT with 200 µl of label-mix, which consisted of PBS pH7.4, 10 µg of Fluorescein (FITC)-labeled polyclonal antibody to *Salmonella* common structural antigens (KPL Europe, Guildford UK) and 10µl of FA Rhodamine counterstain (Difco Laboratories, Detroit, USA). After staining, the cross sections were washed three times with PBS, pH 7.4, before 100µl of mounting solution (Vectashield mounting solution for fluorescence; Vector Laboratories, Inc., Burlingame, CA) was added and slides were covered and sealed. Each slide with cross-sections was analyzed using a fluorescence microscope including CCD-camera.

Messenger RNA and DNA preparation for gene-expression analysis

For gene-expression analysis, 60 seeds were germinated in Hoagland's agar, of which after 3 weeks growth 30 plants were inoculated close to the roots with 10 µl of water and 30 plants were inoculated with 10 µl of 10^7 CFU/ml *S.* Dublin. Every two or three days for a 3-week period (10 time points, including t=0) three water-inoculated plants and three S. Dublin-inoculated plants were harvested by cutting the plants just above the agar surface. At each time point the leafy parts of

each treatment were pooled prior to weighing and subsequently ground in liquid nitrogen and stored at -80°C. Total RNA was extracted from the ground samples using Qiagen Plant RNeasy kit (Westburg, Germany) according to the supplier's protocol (including DNase treatment). The total RNA eluates were aliquoted in several portions before storing at -80°C. Plant mRNA was purified from 45µl of each total RNA sample using the Oligotex mRNA purification kit (Invitrogen). For DNA extraction from ground plant material, the Qiagen plant DNeasy kit (Westburg) was applied as described by the supplier's protocol. The purified DNA was dehydrated / dried using a speed vacuum concentrator, re-suspended in 50µl of milliQ water and stored at -20°C until further use.

cDNA-AFLP differential gene-expression analysis of S. *Dublin colonized lettuce*

Basic principles of *c*DNA-AFLP were followed as described by Bachem et al. (1996; 1998). Each first strand cDNA synthesis reaction was prepared by incubating 10µl of purified mRNA with 2µl of polydT primer (5µM) for 10 min at 70°C, followed by incubation on ice. Next, 4µl of first strand buffer (Invitrogen), 2µl 0.1M DTT, 1µl of 10mM of each dNTP was added to each RNA sample and shortly incubated at 37°C. First strand synthesis was started by adding 1µl of Superscript II RT RNase H^- to each reaction. The sample was incubated for 1 hr at 37°C before ending the synthesis by incubation on ice.

Second strand synthesis was performed for each first strand reaction in a total volume of 150µl containing (Invitrogen), 500nmol of each dNTP, 10 units of *E. coli* DNA ligase, 40 units of *E. coli* DNA polymerase I and 2 units of *E. coli* RNase H, followed by incubation of 2 hrs at 16°C. Then, 2µl of T4 DNA polymerase was added followed by a short incubation of 5 min at 16°C. The final ds cDNA was purified by phenol:chlorophorm:iso-alyl alcohol (1:1:24) and NaAc precipitation. The precipitated DNA was resuspended in 25µl of water.

To perform restriction digestion of the prepared cDNA, 10µl of cDNA sample was added to 40 µl of restriction mix, containing a final concentration of 1xRL-buffer with 10 units of *Mse*I and 50 units of *Eco*RI. The mix was incubated overnight at 4°C.

Subsequently, 20µl of cut cDNA sample was added to 30µl of ligation mix, with a final concentration of 5pmol of *Eco*RI-adapter, 50pmol of *Mse*I-adapter, 1xT4 DNA ligase buffer (Invitrogen), and

containing 5 units of T4 DNA ligase. Ligation was performed by incubating the ligation sample for 90 min at 37°C. The ligated cDNA samples were stored at -20°C upon further use.

Pre-amplification was performed using primers directed to the adapters that were ligated to the cDNA (zero-reaction). Each PCR reaction of 25µl consisted of PCR buffer (10 mM Tris/HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 150nmol of each dNTP, 63pmol of each primer EcoRI00 (5' GACTGCGTACCAATTC 3') and primer Mse00 (5' GATGAGTCCTGAGTAA 3'), 1 unit of Taq polymerase (Gibco BRL) and 5µl of ligated cDNA sample. The reaction mix was incubated for 2 min at 94°C, followed by 35 cycles of respectively 30 sec at 94°C, 30 sec at 56°C and 90 sec at 72°C. Selective PCR was performed as described above, using the primers 33P-kinated EcoRI19 (3' +2 overhang; GA) and Mse11 to Mse26 (+2 nucleotides 3' overhang, each possible combination) with 5 µl of a 50 times dilution of pre-amplification product in a total volume of 20µl. Primer EcoRI19 was kinated to gamma-33P-ATP using polynucleotide kinase prior to PCR. The PCR profiles were as follows: 1 cycle of 94°C for 30 sec, 65°C for 30 sec, 72°C for 1 min, followed by 12 cycles of 94°C for 30 sec, 65°C [-0.7°C/cycle] for 30 sec, 72°C for 1 min, and 23 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min. PCR products were size fractionated on a 5% poly-acrylamide gel and run for 1.5 hrs at 80W.

Isolation of differentially expressed gene fragments and confirmation by RT-PCR

AFLP gels were vacuum blotted and dried on Whatmann 3MM paper for 1 hr and subsequently exposed to X-ray films for 2 weeks. After film development bands of interest were selected and cut from the blotted gel on Whattman paper.

The small paper cuttings were stored in a microtiterplate with 100µl of RNase free water and heated for 5 min at 95°C to elute the DNA from the paper. Next, 5 µl of eluted sample was amplified again by PCR (according to the zero-reaction protocol). Samples were analyzed on gel and sequenced using primer E00. Each transcript-derived fragment (TDF) sequence was compared against all sequences in the non-redundant database using the tBlastX program with the EMBL Database, and TIGR EST library of *L.sativa* and *A. thaliana*.

To allow gene confirmation and expression profiles by RT-PCR, primer sets were designed based on alignments of TDFs with the most probable sequence hits from the EMBL database and TIGR-EST database. Each primer set was designed such that one primer was located inside both sequences of the alignment, and one primer was located outside the TDF, but inside the sequence obtained from the TIGR-EST database. Next to that, other primer sets were designed based on specific lettuce genes related to plant-pathogen interaction, namely pathogenicity-related gene1 (PR1), gene 4 (PR4) and gene 5 (PR5) and defender against apoptotic death (DAD-1). All primer sequences and corresponding genes are displayed in table 1.

In each case the first strand cDNA synthesis was performed using the reverse primer and MuLV-reverse transcriptase. Each 30µl target-specific PCR-reaction consisted of PCR buffer (10 mM Tris/HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 100nmol of each dNTP, 167pmol of each target-specific primer, 1.25 units of Taq polymerase (Gibco BRL) and 0.25 µl of cDNA sample. The reaction mix was incubated for 2 min at 94°C, followed by 35 cycles of respectively 15 sec at 94°C, 30 sec at 58°C and 1 min at 72°C and finished at 10°C for 10 min.

All amplification products were analyzed by electrophoresis using a 1% pronarose gel containing 0.5 µg / ml of EtBr. The intensity of the resulting bands was normalised for each gene-specific primer set using the most intensive band as 100% expression level to allow proper comparisons of the time series of both non-inoculated and inoculated samples.

Results

Plant surface disinfection efficiency

To investigate the presence of *S.* Dublin inside plant tissues, the Salmonellae on the plant surface must be removed very efficiently without killing the bacteria inside the plant. To this extent the efficiency of ethanol 70% was evaluated for surface disinfection of the leafy parts of plants that were inoculated directly on the leaves with *S.* Dublin. On average 5.6 (±1.0) $x10^3$ *S.* Dublin CFUs were recovered after disinfection and 2.9 (±0.1) $x10^6$ CFUs were obtained when no disinfection was applied to the *S.* Dublin-inoculated leaves. From these results the surface disinfection efficiency was determined as 99.81% (± 0.26%).

Colonization of lettuce grown in manure-amended soil by Salmonella Dublin

Since Salmonellae are frequently isolated from bovine manure, it was hypothesized that lettuce plants grown on manure-amended soil can be colonized by these Salmonellae. To investigate the prevalence and degree of infection of lettuce plants with *S.* Dublin, lettuce seeds were applied to soil that was amended with non-inoculated manure or *S.* Dublin inoculated manure. From the seeds applied on soil amended with non-inoculated manure, 61 out of 74 seeds germinated. In soil amended with *S.* Dublin inoculum 56 out of 74 lettuce seeds germinated. This difference was not significant (Chisquare=1.02). The mean weight of the leafy parts of the plants grown for six weeks on *S.* Dublin inoculated manure / soil mixture was 0.52 g ± 0.17 g and the mean weight of the leafy parts of the plants grown for six weeks on non-inoculated manure / soil mixture was 0.57 g ± 0.15 g. Using these data, no significant difference was observed between both treatments using analysis of variance (p=0.153).

The prevalence of *S.* Dublin found in association with the leafy parts of lettuce plants was 27% (15 out of 56 plants). The wash fraction of 15 sampled plants also contained *S.* Dublin, indicating that 27% of the plants were in each case colonized above soil with loosely attached *S.* Dublin. Moreover, three surface disinfected plants were positive for *S.* Dublin (5%), suggesting the presence of *S.* Dublin inside the plant tissue. From these internally colonized plants in two cases also the wash fraction was positive for *S.* Dublin, which indicated the presence of *S.* Dublin also on

the plant surface. From this set the number of *S.* Dublin CFU recovered from the surface disinfected plants ranged from 75 CFU to 1275 CFU per plant. In addition, one non-disinfected plant was also positive for *S.* Dublin, indicating the presence of internal and / or external colonization of the plant by *S.* Dublin. These results suggest that lettuce plants can be colonized by Salmonellae, in our case *S.* Dublin, if grown on soil amended with contaminated manure.

Colonization of lettuce grown in Hoagland's agar by Salmonella Dublin

To study the colonization of lettuce by *S.* Dublin in time, lettuce seeds were germinated on Hoagland's agar and after three weeks the plants were carefully inoculated at the roots with *S.* Dublin. The number of plant-associated *S.* Dublin CFU varied greatly over time among colonized plants, which led to a high S.E.. Therefore no correlation between number of *S.* Dublin CFU and time post-inoculation was obtained. (Table 2). Yet, a large difference in total number of *S.* Dublin CFU was found between the disinfected (mean of 3808 ± 1643 CFUs per plant) and non-disinfected plants (mean of 49582 ± 30012 CFUs per plant) (Table 2). Taking into account the surface disinfection efficiency (99.81%), in this experiment a maximum of 94 CFUs (0.19% of 49582 CFUs) was considered false positive among the disinfected plants. This is appr. 40-fold lower than the average number of CFUs found inside the disinfected plants.

Based on prevalence of *S.* Dublin in association with lettuce plants in the time series tested, all but 3 non-disinfected sprouts were positive (Table 2). In total 17 out of 54 disinfected plants were below the threshold level of 94 CFUs, which indicated that 31% of all 54 disinfected plants was false positive. Still, at least 43% of the 54 disinfected plants were confirmed positive for *S.* Dublin, taking into account the false positive threshold of 94 CFUs. From these results *S.* Dublin appeared more present at the outer surface compared to the inside of the plants (mean ratio of 13:1). The degree of colonization in time with *S.* Dublin inside the lettuce seedlings ranged from 100 CFU up to 4.4×10^4 CFU (potential false positives are excluded), whereas the degree of colonization both inside and outside the plants ranged from 2 CFU up to 1.1×10^6 CFU per plant.

Localization of S. Dublin in association with lettuce plants

To evaluate if *S.* Dublin was able to colonize the plant up to the leaves, different plant parts were tested by grinding the tissues and subsequent plating on selective Hektoen agar. From this experiment *S.* Dublin was found associated with lettuce plants, mainly from the root-stem transition point up to the leaves, but not in the leaves (data not shown).

Then, to determine the possible point of entrance of *S.* Dublin in lettuce plants, cross sections of colonized plants were analyzed by fluorescence microscopy (Figure 1). Analyses of the cross-sections revealed strong growth of *S.* Dublin on the root surface (1A and B) and near emerging lateral roots (1C). Moreover, internalization was observed via the intercellular spaces between epidermal cells (1D). *S.* Dublin bacteria were found at the cortex within the parenchyma tissue (1E), either still attached to epidermal cells or spreading further through the parenchyma tissue. In few cases *S.* Dublin was also found attached to endodermal cells, inside the pericycle (1E) or even inside the vascular system (1F), which suggests the bacterium might be able to pass the endodermis and potentially spread upwards via the vascular system.

Symptom development and biomass of lettuce grown in Hoagland's agar

Lettuce plants responded to the presence of *S.* Dublin in and on the plant tissue by showing reduced root formation and stunted growth. With respect to biomass, up to 10 days post inoculation (dpi) no clear differences in plant growth were observed between non-inoculated and inoculated plants (Figure 2). From 12dpi onwards, growth of lettuce plants inoculated at the roots with *S.* Dublin was significantly reduced compared to the non-inoculated lettuce plants. Fitting the data sets using logistic regression, separate curves for the healthy plants (R^2-value of 0.98) and the inoculated plants (R^2-value of 0.97) were obtained (Figure 2). Linear regression of all data (R^2-value of 0.80) resulted in a worse fit than separate data sets (healthy: R^2-value of 0.95, inoculated: R^2-value of 0.94). A difference in slope (factor 2) of the linear regression curves was obtained which suggests a significantly stronger growth of healthy plants compared to inoculated plants when grown in Hoagland's agar.

To determine if the stunted growth was specifically related to the colonization by *S.* Dublin, morphological changes due to the presence of *S.* Dublin were compared to those in the presence of

E. coli JM109 and to healthy, water-inoculated plants. The non-pathogenic *E. coli* was used to ensure that the symptoms observed were specific to the presence of *S.* Dublin and not due to inoculation side effects or presence of bacteria in general. From sixteen days post-inoculation onwards, yellow spots appeared on the leaves of 8 *S.* Dublin-inoculated lettuce plants. The root basis of these plants became narrowed just below the transition point from root to stem. At 19 dpi, the leaves were strongly yellowed, whereas two plants remained healthy. However, with *E. coli* JM109 only four plants showed only very slight yellowing and stunting at 20 dpi, while six plants remained healthy. During the experiment all ten water-inoculated plants remained healthy. No bacterial growth was detected in Hoagland's agar.

Non-parametric analysis (Kruskall-Wallis test) of the total number of remaining healthy lettuce plants revealed a significant difference between all three treatments ($p=0.008$). Comparing two treatments for the number of healthy plants, a significant difference ($p=0.002$) was observed between water-inoculated plants and *S.* Dublin inoculated plants. Water-inoculated plants compared with *E.coli* JM109 inoculated plants showed no significant difference ($p= 0.057$). This indicated that the observed morphological changes of the lettuce plants were induced by the presence of *S.* Dublin, and not due to the presence of a bacterium in general (in our case *E. coli*) or depletion of nutrients (healthy controls).

As an additional control, the different treatments were also compared based on the biomass of the healthy, i.e. surviving plants, indicating no significant difference ($p=0.141$) between all plants of the three treatments. This suggested the remaining healthy plants of the three treatments (two *S.* Dublin-treated plants, six *E. coli*-treated plants, ten water-treated plants) were not colonized or influenced by the bacteria added close to the roots.

Identification of lettuce genes differentially expressed due to Salmonella colonization

From the physiological response of lettuce plants to *S.* Dublin colonization and the presence of *S.* Dublin in and outside lettuce plant tissues, it was suggested that the plant also responded on a molecular level. To determine the molecular response of lettuce during colonization by *S.* Dublin in time, gene expression analyses were performed using cDNA-AFLP. The transcript-derived

fragments (TDFs) displayed from cDNA-AFLP were obtained from 16 primer sets (EcoRI-T and Mse-NN) that were tested. On average for each primer set 100 to150 bands were observed, resulting in approx. 2000 fragments that were analyzed. Although the majority of bands revealed no differential expression profiles between non-inoculated and Salmonella-inoculated plants, also discriminative bands were found. In total 170 bands were selected from cDNA-AFLP, of which 90 bands showed differential expression profiles between both treatments (non-inoculated and inoculated), in time. The selected 170 bands were sequenced of which 68 consisted of more than one sequence, thus leaving 102 sequences valid for further analysis. The 102 sequences were blasted using tblastx against EST databases from *L. sativa* (22.185 EST) and *A. thaliana* (62.010 EST) (TIGR-gene indices). On the basis of sequence homology, these 102 TDFs were grouped in 12 categories of putative function followed by classification according to Mahalingam *et al* (2003; 31) (Table 3). Comparing differentially expressed TDFs versus generally expressed TDFs, an increase was observed for the categories related to plant disease / defense, transport, signal transduction and hypothetical proteins (table 3). A reduction in gene expression was observed for plant metabolism, and genes with unknown functions.

Specific gene-expression due to the presence of *S.* Dublin was confirmed by evaluation of PCR primer sets directed to DNA sequences coding for proteins known to be related to plant stress (Table 1). The expression of the plant stress-related genes DAD1-gene (33), PR1-gene (10), PR4-gene (38) and PR5-gene (42) was induced by the presence of *S.* Dublin at 2dpi (Table 4). This suggested that the plant defense mechanism was activated by the presence of *S.* Dublin in association with the plant.

Next, 11 tentative differentially expressed genes obtained from cDNA-AFLP were analyzed with RT-PCR, of which five genes (Probable threonine ammonia-lyase, Receptor protein kinase-like protein, Beta-expansin 1 precursor, Pyruvate dehydrogenase kinase, and Phospholipid hydroperoxide glutathione peroxidase) eventually appeared to be equally expressed in time with both treatments or did not reveal a significant difference (not shown), and were therefore excluded from further analyses. From the other six genes, namely *NO APICAL MERISTEM* (NAM)-like protein -gene, Oxygen-evolving enhancer protein 3-2 chloroplast precursor (OEE3)-gene, pathogenicity-related

protein 1, Secretion 1-family transport protein gene, Secretion 6 transport protein gene and bHLH016 transcription factor protein gene, differential gene expression patterns were observed between non-inoculated plants and *S.* Dublin-inoculated plants (Table 4). The pathogenicity-related protein1-gene was based on the PR1-gene sequence of *A. thaliana* and gave the same results (as expected) as obtained with the primer set PR1. The NAM-like protein -gene, related to resistance and cell death, revealed an increase in expression only at 2 dpi followed by a reduction in expression compared to the NAM-gene expression of non-inoculated plants. The expression of the OEE3-gene (energy function) was consistently high until 6 dpi, but then reduced dramatically in time to undetectable levels. The sec1 and sec 6 transport protein genes are both involved in intracellular mRNA transport and cell proliferation, which each showed a consistent expression in time with the untreated plants. But with the *S.* Dublin inoculated plants, the gene expression of both genes was reduced to undetectable levels after 4 dpi and 10 dpi, respectively. In addition to this, the expression of the bHLH016 transcription factor protein gene suggested a high level of expression during the early stages of colonization, but slowly reduced by intensity in time. For non-inoculated plants, the expression of this gene was moderate at first, increasing in intensity in time.

Discussion

This study investigated the physiological and molecular response of *L. sativa* cultivar Tamburo to *S.* Dublin. Lettuce plants were colonized both endophytically and epiphytically when lettuce seeds were germinated and sprouted on *S.* Dublin-inoculated manure amended soil (prevalence of 27%). Lettuce grown under sterile conditions was even more susceptible to colonization by *S.* Dublin via the roots (prevalence of 43%) than when grown on soil. With both approaches *S.* Dublin was mainly present on the plant surface but also endophytically, at a ratio of 13:1.

These results rely on the surface disinfection efficiency obtained from artificially inoculated leaves. This does not fully reflect a completely realistic situation. Naturally occurring bacteria are able to form a protective biofilm on the leaf surface that prevents the penetration of disinfectants and subsequent lysis of the bacterial cells (29). This would imply a less efficient surface disinfection in case of naturally infected plants. On the other hand, biofilms are only (partially) protective against very mild disinfectants like chlorine, but are likely much less, or even not protective against 70% ethanol. Moreover, from the tissue cross sections tested with fluorescent microscopy it was evidenced that *S.* Dublin was present both inside and outside the plant, which suggests the *S.* Dublin CFUs found after surface disinfection were indeed endophytically present in the lettuce plant tissue.

The invasion process observed in this research was similar to the invasion of barley with *S.* Typhimurium (28). It was suggested that the invasion proces of *S.* Typhimurium for colonizing plants is similar to that of plant pathogens (28), characterized by a three-phase process of *Ralstonia solanacearum* infecting hydroponically grown tomato plants (43). First the root surface is colonized, followed by infection of the vascular parenchyma and then invasion of the xylem. This three phase process was also observed in this study.

S. Dublin was able to colonize the lettuce plant endophytically and epiphytically, both under sterile growing conditions and in manure-amended soil. Epiphytic movement of *Salmonella* cells from the soil or medium to the aerial portions of the plant was allowed via capillary forces to retain a non-

disturbed colonization and plant-microbe interaction. *S.* Dublin first colonized the root surface reaching a high density of bacterial cells around naturally present openings or wounds. This is in line with demonstrated bacterial growth and rhizosphere colonization stimulated by root exudates (6, 8) and the observed biofilm formation with the lettuce cross-sections described in this paper. Subsequently, invasion occurred via wounds that allowed the bacteria to colonize the roots intercellularly (9, 39), but also via intercellular spaces between epidermal cells. Indeed, *S.* Dublin was found in the parenchyma tissue and inside the pericycle, attached to, and inside, the vascular system.

Typically, the stems of sterile grown plants appeared constricted at the root-stem transition point several days after inoculation of the roots with *S.* Dublin. Lettuce may have responded in a hypersensitive manner to intercellular presence of *S.* Dublin. This may have led to reduced nutrient flow, leaf yellowing and finally plant death, herewith indicating that *S.* Dublin might be pathogenic to lettuce under these conditions. A critical point would be that the cell density used for inoculation was rather high with the soil experiments. Indeed, 10^7 cells / g are not often found in the environment. However, the level of inoculum was applied as a worse case scenario to provide insight in the colonization efficiency of *S.* Dublin with soil-grown plants. To what extent lettuce is still colonized at lower *S.* Dublin cell densities, needs yet to be determined.

In view of the symptoms on plants inoculated with *S.* Dublin, inoculated plants apparently reacted physiologically to colonization by this human pathogen. We also demonstrated, for the first time by cDNA-AFLP analysis, that plant genes were differentially expressed between *S.* Dublin-inoculated and non-inoculated plants. An increase in expression of pathogenicity related genes was observed, which suggest a similar response of lettuce to colonization by *S.* Dublin as with plant pathogenic bacteria. The expression profiles of at least nine genes were strongly associated with the colonization of lettuce by *S.* Dublin. Next to four genes DAD1 (33), PR1 (10), PR4 (38) and PR5 (42) that are known to be related to plant stress, also five other genes were obtained from cDNA-AFLP that had differential profiles between colonized and non-colonized plants, namely NAM-like

protein gene (13), OEE3 gene (40), PR1-gene (10), Sec1-family transport protein gene (2), Sec6 transport protein gene (46), and bHLH016 transcription factor gene (20).

The NAM-like protein is involved in shoot development and leaf formation of *Petunia* (13). In line with this, plant growth was stunted when colonized with *S.* Dublin and the gene expression was reduced in time compared to healthy plants. For bHLH transcription factor protein genes, the *Arabidopsis* genome encodes for at least 150 putative bHLH class transcription factors, of which many play key roles in phytochrome signal transduction (20). These transcription factors are suggested to primarily act more as negative regulators than positive regulators of the phytochrome signalling (12). Moreover, these bHLH proteins are found to interact specifically with phytochromes. For example, phytochrome-interacting factor 3 (PIF3) mainly acts as a negative regulator in the phytochrome B pathway, but as a positive regulator of anthocyanin and chlorophyll accumulation (12). The expression profile of the bHLH016 gene identified in this study showed a decrease in time for the colonized plants, but an increase in time for non-inoculated plants. This difference can explain the development of symptoms such as leaf yellowing, implying a reduction of chlorophyll production, which is in line with the expression profile. In addition, yellow leaves also lead to less phytochrome translocation, which is induced by negative regulation of the bHLH proteins.

The Oxygen-evolving enhancer protein 3-2 chloroplast precursor (OEE3) is one of the three OEEs (OEE1, OEE2, OEE3), which are nuclear-encoded chloroplast proteins that are bound to photosystem II (PSII) (40). Reduction of the gene expression has a direct influence on photosystem II, leading to a reduction of photosynthesis (40). This might be a secondary effect of colonization with *S.* Dublin, since the bacteria are thought to narrow the stem and herewith reducing the nutrient flow dramatically.

The expression profiles of the PR1 gene are similar to the previously published expression profile of PR1 gene of *Medicago* when colonized with *Salmonella* spp. (11), which implies a salicylic acid directed defence mechanism of the plant upon colonization with *Salmonella* spp. These results would suggest a pathogenicity related response of lettuce to colonization by *S.* Dublin.

Interestingly, the expression of genes (sec1 and sec6) involved in the regulation and formation of the actin cytoskeleton (2, 46) was strongly affected during colonization. Inhibition of SNARE (soluble

NSF attachment protein receptor) regulatory proteins consequently block mRNA transport by depolarization of the actin cytoskeleton, which will eventually lead to cell death. This might explain the fact that leaf yellowing (and eventually plant death) was observed from the colonized plants. The onset of the secretory block is thought to be activated by a specific signal that influences the actin regulatory machinery. The nature of this signal is not yet known. However, taking into account the results described in this paper (a strong reduction of the sec1 and sec6 gene expression in time, in contrast to the healthy plants) it might be that the signal is activated by certain bacteria during colonization of the host plant. Since *Salmonella* spp. are known to change / disrupt the actin cytoskeleton prior to invasion of mammalian cells, it might very well be that specific secretory proteins of *Salmonella* spp. are responsible for the blocking of the SNARE regulatory proteins of lettuce. According to this hypothesis the expression profiles of these genes might be related to a more specific than general response of lettuce upon colonization by *S.* Dublin. In that case these genes would be designated as potential marker genes, which is especially of great interest with respect to food safety. This theory should be investigated further to better understand the molecular interaction between lettuce and *Salmonella* spp. during colonization and to clearly identify the presence of such marker genes.

In conclusion, previous studies postulated *Pseudomonas auruginosa* and *Staphylococcus aureus* to be plant pathogenic (36). Whether *S.* Dublin could also be designated as a pathogen for lettuce cv Tamburo is not fully validated. Under sterile growing conditions symptoms (leaf yellowing, stunting) were observed, whereas no symptoms could be observed on lettuce grown in soil. This might indicate that lettuce Tamburo is susceptible for *S.* Dublin. However, this does not mean that all lettuce cultivars would be equally susceptible to *S.* Dublin, nor that all strains of *Salmonella enterica* would equally efficient colonize lettuce. A lettuce cultivar - *Salmonella* strain interaction study would be very interesting and valuable for agriculture and society, in order to reduce, or even prevent the risk of disease outbreaks related to the consumption of fresh produce.

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Figure 1:

Fluorescence microscopy of cross sections of the root-shoot transition region of lettuce plants colonized with *Salmonella* Dublin. The *S.* Dublin was visualized using a FITC-labeled antibody directed to *Salmonella enterica* in the cross sections, indicated by arrows. The bacteria were clearly detected on the root surface (A and B) and at emerging lateral roots (C). Internalization was observed via the intercellular spaces between epidermal cells (D). Endophytically present *S.* Dublin was observed in the parenchyma tissue (1E), attached to the endodermal cells and inside the pericycle, and inside the vascular system (1F).

Figure 2:

Logistic and linear regression of plant weight of non-inoculated plants and plants inoculated at the roots with *Salmonella* serovar Dublin versus time of sampling.

Table 1:

Primer sequences for RT-PCR of plant genes

primer	Sequence 5' – 3'	Gene homology	Accession number[a]	p
FPPR1	GGTACACGGCTTATGGTCAAACAG	Pathogenicity-related protein 1	BQ846446	0
RPPR1	TCCATAAGCCACCAAATCAGCATC			
FPPR4	GATCTCTTAGCCACAAACCCAACC	Pathogenicity-related protein 4	BQ874271	0
RPPR4	AACCGGACCCGCTGACCTATCT			
FPPR5	TGCCTCGGAGATTAGTGGGGATAG	Pathogenicity-related protein 5	BQ869968	0
RPPR5	CGCCGTCAATACCGCTTTTACA			
FPDAD1	GACGGCGACGACGAAAGATGAT	Defender against apoptotic death protein 1	BQ987261	0
RPDAD1	GCGGTGAAGACGGCGAACA			
FPTHRE	GCTATAGGCCTTGCTGCTGTTCTC	Probable threonine ammonia-lyase	BQ870155 / TC12830	$8.5e^{-14}$
RPTHRE	GGTTTCATGGGCCTCCTTATTT			
FPEXP	GGAATCACATCCCTTGCTGACAGA	Beta-expansin 1 precursor (At-EXPB1) (Ath-ExpBeta-1.5)	BQ867493	$6.6e^{-18}$
RPEXP	TAACCGCGGCGTACTGAACATC			
FPOXY	ACAGCTCCACCCGTTTGACACC	Oxygen-evolving enhancer protein 3-2 chloroplast precursor (OEE3)	BQ995404 / TC12174	$6.6e^{-36}$
RPOXY	TCTTTCGCCGATTCCTTTACACG			
FPREC	GCAAGGACCAGTAGGCGAGGTGTAC	Receptor protein kinase-like protein	BQ867195	$1.8e^{-09}$
RPREC	ACAACCCCAAAAGAATAAACATC			
FPNAM	TCAAGTCCCGGAAGTAAAAGAG	NAM-like protein	BQ864249 / TC12743	$1.9e^{-05}$
RPNAM	ACCTGATGATGGATAAGAAATAGC			
FPPAT	TCCGACGTCAAAAAGAAGATAAC	Pathogenesis-related protein 1 precursor (PR-1)	BQ846446	0.95
RPPAT	CTTACACACACATATTCATTCA			
FPPYR	TCGAAGGCTCCGGTGATAAAAT	pyruvate dehydrogenase kinase	BQ870017 / TC11537	$6.4e^{-29}$
RPPYR	NTGAGAAAGGGTTGCGTGTTG			
FPPHO	GCCCCTAAAACCCCTCCTCT	phospholipid hydroperoxide glutathione peroxidase	TC9259	$1.4e^{-05}$
RPPHO	AACCCCTCCTTCTAGCGATTCA			
FPBHL	CCGAACGGAAAAGAAGAGACAAG	putative bHLH transcription factor bHLH016	TC9488	$1.6e^{-07}$
RPBHL	GTGGACCACAGGTTTGATTTTGC			
FPSEC6	TGATAAAGTCCAGCCTCCAAAAT	SEC6	TC14586	0.86
RPSEC6	GCAAGATCATAGCATCTCAAGTTGT			
FPSEC1	AATGGTTGAATCCGCGTTGAGAG	SEC1-family transport protein SLY1	TC9658	$1.0e^{-12}$
RPSEC1	TTAGGCAGGAGCAGAAGCAGAAGG			

[a]Accession numbers of genes in TIGR gene indices, that is linked to the non-redundant EMBL database

[b]The smallest sum probability (p-value) according to EMBL genbank

Table 2:

Colonization of lettuce by *Salmonella* Dublin over time

Sampling day	disinfected[a]			non-disinfected[a]		
	prevalence[b]	CFU / plant[c]		prevalence	CFU / plant	
		Mean	SE[d]		Mean	SE
0	0/6	0	0	0/6	0	0
2	2/6	297	43	6/6	14803	2641
4	2/6	2094	455	5/6	7756	2851
6	2/6	2413	324	5/6	18139	2919
8	2/6	1678	318	4/6	2377	606
10	2/6	5284	1200	6/6	10624	1895
12	5/6	10403	3118	6/6	6035	979
14	1/6	1463	0	6/6	13843	2347
16	2/6	1138	243	6/6	34070	7305
18	5/6	1661	363	6/6	317531	68920
total	43%	3808	1643	93%	49582	30012

[a]*Surface disinfected and non-disinfected lettuce plants tested for presence of Salmonella spp. in time*

[b]*The number of plants positive for Salmonella spp. / total number of plants tested, in time*

[c]*The mean number of Salmonella spp. CFU per plant that were in association with the surface disinfected and non-disinfected lettuce plants, in time.*

[d]*The standard error of the mean number of Salmonella spp. CFU found on the plants per timepoint.*

Table 3:

Contribution of functional groups to general and differential gene-expression

General[a]			Differential[b]			difference in expression[c]
Functional groups	number of genes[d]	%[e]	Functional groups	number of genes	%	
cell growth/devision[f]	1	2%	cell growth/devision	0	0%	=
cell structure	1	2%	cell structure	0	0%	=
disease/defence	6	11%	disease/defence	9	21%	+
energy	9	16%	energy	8	19%	=
Transport	7	13%	transport	7	16%	+
metabolism	12	21%	metabolism	3	7%	-
protein degradation/storage	1	2%	protein degradation/storage	1	2%	=
protein synthesis	1	2%	protein synthesis	1	2%	=
signal transduction	3	5%	signal transduction	5	12%	+
transcription	1	2%	transcription	0	0%	=
hypothetical protein	3	5%	hypothetical protein	6	14%	+
Unknown	10	18%	unknown	3	7%	-
total:	56		Total:	43		

[a]Generally expressed genes separated by function

[b]Differentially expressed genes separated by function

[c]Displays a decrease (-), increase (+) or equal (=) number of genes being expressed for each functional group[f] when comparing the percentage of generally and differentially expressed genes per functional group.

[d]Total number of genes per functional group found after EMBL and TIGR database searching, and [e]the percent contribution to the transcriptome

Table 4:

Gene expression profiles of transcript derived genes and pathogenicity related genes

Genes[a]	Treatment[b]	Normalised gene expression in time (dpi)[c]									
		15 min	2	4	6	8	10	12	14	16	18
Defender against cell death-protein-gene	-	0.9[d]	0.8	0.7	0.6	0.6	0.7	0.3	0.4	0.7	0.8
	+	0.8	1.0	1.0	0.8	1.0	0.6	0.6	0.5	0.4	0.2
Pathogenicity related protein 1-gene	-	0.3	0.3	0.4	0.4	0.6	0.7	0.5	0.2	0.3	0.4
	+	0.8	1.0	1.0	1.0	0.6	0.2	0.8	0.4	0.0	0.2
Pathogenicity related protein 4-gene	-	0.2	0.3	0.5	0.5	0.3	0.3	0.8	0.3	0.5	0.8
	+	0.3	0.7	0.3	0.7	1.0	0.7	0.7	0.5	0.3	0.3
Pathogenicity related protein 5-gene	-	0.0	0.3	0.6	0.5	0.3	0.3	0.8	0.3	0.4	0.8
	+	0.6	1.0	0.4	0.6	0.8	0.4	0.6	0.4	0.2	0.4
No apical meristem-like protein-gene	-	1.0	1.0	1.0	0.8	1.0	1.0	0.4	1.0	1.0	1.0
	+	0.3	1.0	0.7	0.3	0.7	1.0	0.3	0.0	0.0	0.0
Oxygen evolving enhancer protein-gene	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.8	1.0
	+	1.0	0.7	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sec1 transport protein-gene	-	0.2	1.0	0.8	0.8	1.0	0.6	0.6	0.6	1.0	1.0
	+	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sec6 transport protein-gene	-	0.8	0.8	1.0	0.8	0.8	0.8	0.6	1.0	1.0	1.0
	+	1.0	1.0	1.0	1.0	0.3	0.3	0.5	0.3	0.0	0.0
bHLH16 transcription factor protein-gene	-	0.4	0.4	0.6	0.6	0.6	0.8	0.8	1.0	1.0	1.0
	+	1.0	1.0	0.9	0.9	0.7	0.4	0.4	0.1	0.0	0.0

Gene-expression in time measured by Reverse Transcriptase-PCR for various genes with Salmonella Dublin- inoculated plants (+) and water-inoculated plants (-). The level of expression was normalised for each gene ranging from high (green) to no expression (red).

[a]Genes analyzed with for level of gene-expression, in time

[b]Treatment of plants that were analyzed in time. Plants were inoculated at the roots with S. Dublin (+) or with water (-).

[c]Sampling times of lettuce plants for gene-expression analysis

[d]Normalised level of gene-expression obtained after Reverse Transcriptase-PCR and gel analysis. Normalisation was performed for each gene separately based on band intensity on gel.

Figure 1

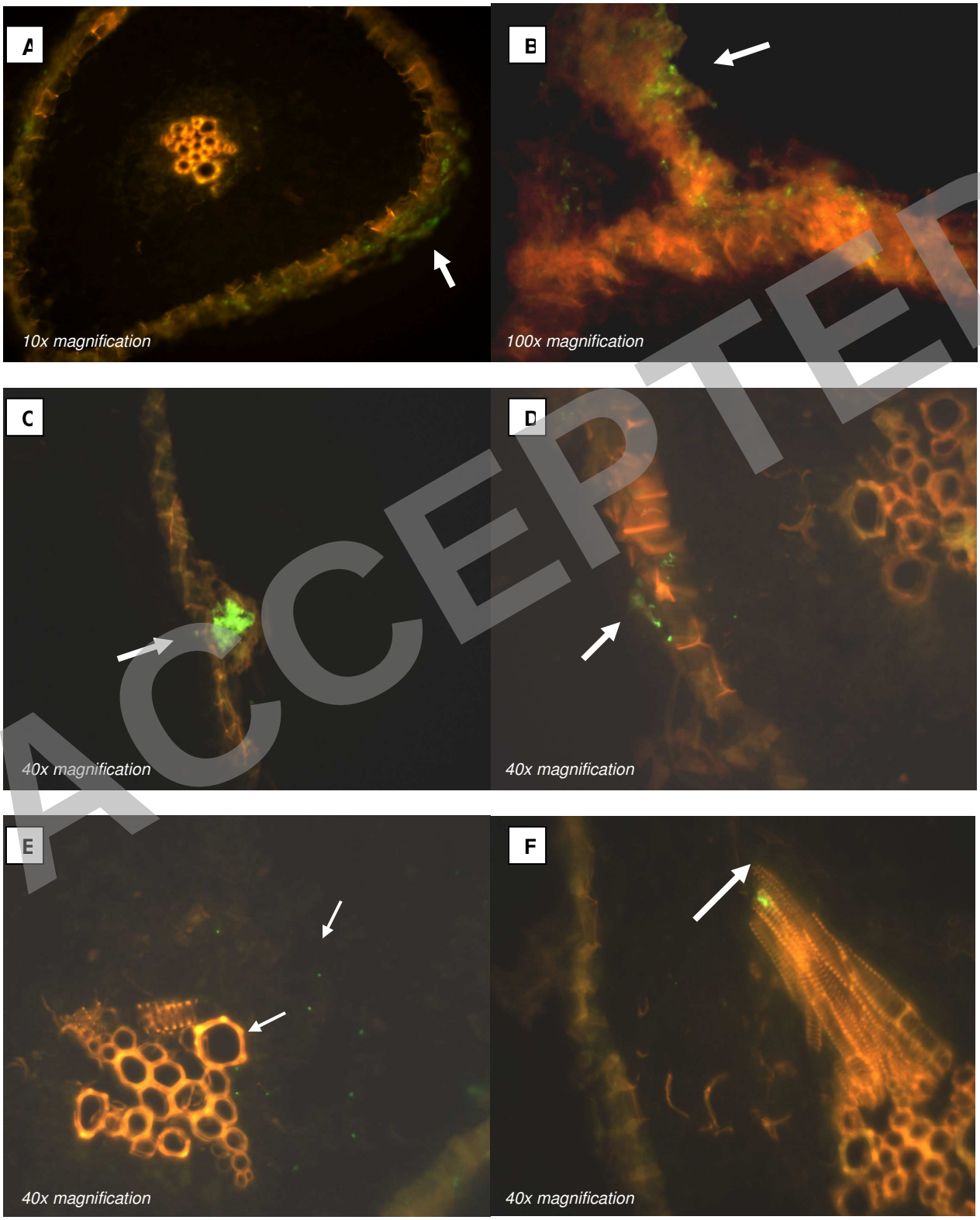
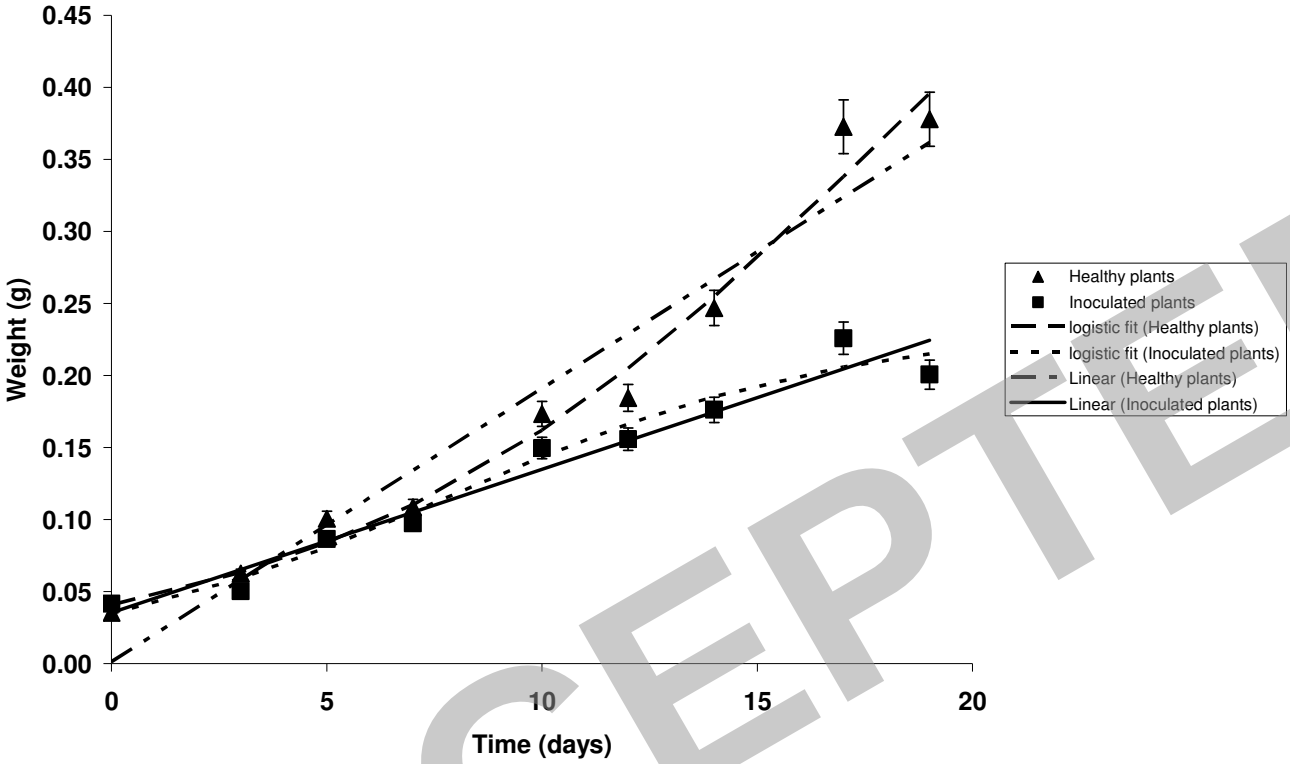


Figure 2:



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